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United States Patent and Trademark Office

March 24, 2000

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APPLICATION NUMBER: *60/161,997* — 28-10-1999
FILING DATE: *October 28, 1999*
PCT APPLICATION NUMBER: *PCT/US00/03331*

By Authority of the
COMMISSIONER OF PATENTS AND TRADEMARKS

N. Williams

N. WILLIAMS
Certifying Officer

PRIORITY DOCUMENT
SUBMITTED OR TRANSMITTED IN COMPLIANCE WITH RULE 17.1(a) OR (b)

PROVISIONAL APPLICATION COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION under 37 CFR 1.53(c).

Docket Number CHIR-0214		Type a plus sign (+) inside this box→	+

INVENTOR(S)/APPLICANT(S)

LAST NAME	FIRST NAME	MIDDLE INITIAL	RESIDENCE (CITY AND EITHER STATE OR FOREIGN COUNTRY)	CITIZENSHIP (if known)
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TITLE OF THE INVENTION (280 characters max)

OIL DROPLET EMULSION ADJUVANTS COMPRISING CpG OLIGONUCLEOTIDES AND USES THEREFOR

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ENCLOSED APPLICATION PARTS *(check all that apply)*

XX Specification *Number of Pages* 35
XX Drawing(s) *Number of Sheets* 4
_ Small Entity Statement
_ Other *(specify)*

METHOD OF PAYMENT *(check one)*

XX A check or money order is enclosed to cover the Provisional filing fee

_ The Commissioner is hereby authorized to charge filing fee and credit Deposit Account Number: 23-3050

PROVISIONAL FILING FEE AMOUNT ($)150.00

The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

XX No.

_ Yes, the name of the U.S. Government agency and the Government contract number are: _____

Respectfully submitted,

SIGNATURE Paul K Legaard

Date: October 28, 1999

REGISTRATION NO. 38,534 (if appropriate)

TYPED or PRINTED NAME Paul K. Legaard

_ Additional inventors are being named on separately numbered sheets attached hereto.

PROVISIONAL APPLICATION FILING ONLY

A1P10V

DOCKET NO. : CHIR-0214 **PATENT**

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In Re Application of:

John Donnelly, Gary Ott, Derek O'Hagan,
Jina Kazzaz, Mildred Ugozzoli

Serial No.: Not Yet Assigned **Group Art Unit:** Not Yet Assigned

Filing Date: Herewith **Examiner:** Not Yet Assigned

For: OIL DROPLET EMULSION ADJUVANTS COMPRISING CpG OLIGONUCLEOTIDES AND USES THEREFOR

EXPRESS MAIL LABEL NO: EL219292535US
DATE OF DEPOSIT: October 28, 1999

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Washington DC 20231

Sir:

PATENT APPLICATION TRANSMITTAL LETTER

Transmitted herewith for filing, please find

☐ A Utility Patent Application under 37 C.F.R. 1.53(b).

It is a continuing application, as follows:

☐ continuation ☐ divisional ☐ continuation-in-part of prior application number ___/_____.

☒ A Provisional Patent Application under 37 C.F.R. 1.53(c).

☐ A Design Patent Application (submitted in duplicate).

Including the following:

☒ Provisional Application Cover Sheet.

☒ New or Revised Specification, including pages 1 to 35 containing:

☒ Specification

☐ Claims

☒ Abstract

☐ Substitute Specification, including Claims and Abstract.

☐ The present application is a continuation application of Application No. _____ filed _____. The present application includes the Specification of the parent application which has been revised in accordance with the amendments filed in the parent application. Since none of those amendments incorporate new matter into the parent application, the present revised Specification also does not include new matter.

☐ The present application is a continuation application of Application No. _____ filed _____, which in turn is a continuation-in-part of Application No. _____ filed _____. The present application includes the Specification of the parent application which has been revised in accordance with the amendments filed in the parent application. Although the amendments in the parent C-I-P application may have incorporated new matter, since those are the only revisions included in the present application, the present application includes no new matter in relation to the parent application.

☐ A copy of earlier application Serial No. _____ Filed _____, including Specification, Claims and Abstract (pages 1 - @@), to which no new matter has been added TOGETHER WITH a copy of the executed oath or declaration for such earlier application and all drawings and appendices. Such earlier application is hereby incorporated into the present application by reference.

☐ Please enter the following amendment to the Specification under the Cross-Reference to Related Applications section (or create such a section) : "This Application: ☐ is a continuation of ☐ is a divisional of ☐ claims benefit of U.S. provisional Application Serial No. _____ filed _____

_____

_____

_____.

☐ Signed Statement attached deleting inventor(s) named in the prior application.

☐ A Preliminary Amendment.

☒ Four _____ Sheets of ☐ Formal ☒ Informal Drawings.

☐ Petition to Accept Photographic Drawings.

☐ Petition Fee

☐ An ☐ Executed ☐ Unexecuted Declaration or Oath and Power of Attorney.

☐ An Associate Power of Attorney.

☐ An ☐ Executed ☐ Copy of Executed Assignment of the Invention to _____ _____.

☐ A Recordation Form Cover Sheet.

☐ Recordation Fee - $40.00.

☐ The prior application is assigned of record to _____

☐ Priority is claimed under 35 U.S.C. § 119 of Patent Application No. _____ filed _____ in _____ (country).

☐ A Certified Copy of each of the above applications for which priority is claimed:

☐ is enclosed.

☐ has been filed in prior application Serial No. _____ filed _____.

☐ An ☐ Executed or ☐ Copy of Executed Earlier Statement Claiming Small Entity Status under 37 C.F.R. 1.9 and 1.27

☐ is enclosed.

☐ has been filed in prior application Serial No. _____ filed _____, said status is still proper and desired in present case.

☐ Diskette Containing DNA/Amino Acid Sequence Information.

☐ Statement to Support Submission of DNA/Amino Acid Sequence Information.

☐ The computer readable form in this application _______, is identical with that filed in Application Serial Number _______, filed _______. In accordance with 37 CFR 1.821(e), please use the ☐ first-filed, ☐ last-filed or ☐ only computer readable form filed in that application as the computer readable form for the instant application. It is understood that the Patent and Trademark Office will make the necessary change in application number and filing date for the computer readable form that will be used for the instant application. A paper copy of the Sequence Listing is ☐ included in the originally-filed specification of the instant application, ☐ included in a separately filed preliminary amendment for incorporation into the specification.

☐ Information Disclosure Statement.

- ☐ Attached Form 1449.
- ☐ Copies of each of the references listed on the attached Form PTO-1449 are enclosed herewith.

☐ A copy of Petition for Extension of Time as filed in the prior case.

☐ Appended Material as follows: ______________________________.

☒ Return Receipt Postcard (should be specifically itemized).

☐ Other as follows: ____.

FEE CALCULATION:

☐ Cancel in this application original claims _____ of the prior application before calculating the filing fee. (At least one original independent claim must be retained for filing purposes.)

			SMALL ENTITY		NOT SMALL ENTITY	
			RATE	FEE	RATE	FEE
PROVISIONAL APPLICATION			$75.00	$	$150.00	$150.00
DESIGN APPLICATION			$155.00	$	$310.00	$0
UTILITY APPLICATIONS BASE FEE			$380.00	$	$760.00	$0
UTILITY APPLICATION; ALL CLAIMS CALCULATED AFTER ENTRY OF ALL AMENDMENTS						
	No. Filed	No. Extra				
TOTAL CLAIMS	- 20 =		$9 each	$	$18 each	$0
INDEP. CLAIMS	- 3 =		$39 each	$	$78 each	$0
FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM			$130	$	$260	$0
ADDITIONAL FILING FEE				$		$0
TOTAL FILING FEE DUE				$		$150.00

☒ A Check is enclosed in the amount of $ 150.00 .

☒ The Commissioner is authorized to charge payment of the following fees and to refund any overpayment associated with this communication or during the pendency of this application to deposit account 23-3050. This sheet is provided in duplicate.

☐ The foregoing amount due.

☒ Any additional filing fees required, including fees for the presentation of extra claims under 37 C.F.R. 1.16.

☒ Any additional patent application processing fees under 37 C.F.R. 1.17 or 1.20(d).

☐ The issue fee set in 37 C.F.R. 1.18 at the mailing of the Notice of Allowance.

☒ The Commissioner is hereby requested to grant an extension of time for the appropriate length of time, should one be necessary, in connection with this filing or any future filing submitted to the U.S. Patent and Trademark Office in the above-

identified application during the pendency of this application. The Commissioner is further authorized to charge any fees related to any such extension of time to deposit account 23-3050. This sheet is provided in duplicate.

SHOULD ANY DEFICIENCIES APPEAR with respect to this application, including deficiencies in payment of fees, missing parts of the application or otherwise, the United States Patent and Trademark Office is respectfully requested to promptly notify the undersigned.

Date: 28 October 1999

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OIL DROPLET EMULSION ADJUVANTS COMPRISING CpG OLIGONUCLEOTIDES AND USES THEREFOR

FIELD OF THE INVENTION

The present invention is related generally to the field of immune responses and specifically to compositions or combinations of therapeutic and prophylactic compositions, such as, for example, immunogenic compositions, i.e., vaccines.

BACKGROUND OF THE INVENTION

Adjuvants are compounds which are capable of potentiating an immune response to antigens. Adjuvants can potentiate both humoral and cellular immunity. However, it is preferable for certain pathogens to stimulate cellular immunity and, indeed, Th1 cells. Presently used adjuvants do not adequately induce Th1 cell responses, and/or have deleterious side effects.

Currently, the only adjuvants approved for human use in the United States are aluminum salts (alum). These adjuvants have been useful for some vaccines including hepatitis B, diphtheria, polio, rabies, and influenza, but may not be useful for others, especially if stimulation of cell-mediated immunity is required for protection. For example, reports indicate that alum failed to improve the effectiveness of whooping cough and typhoid vaccines and provided only a slight effect with adenovirus vaccines. Additionally, problems such as, induction of granulomas at the injection site and lot-to-lot variation of alum preparations have been experienced.

Complete Freund's adjuvant (CFA) is a powerful immunostimulatory agent that has been used successfully with many antigens on an experimental basis. CFA is comprised of three components: a mineral oil, an emulsifying agent such as Arlacel A, and killed mycobacteria such as *Mycobacterium tuberculosis*. Aqueous antigen solutions are mixed with these components to create a water-in-oil emulsion. CFA causes severe side effects, however, including pain, abscess formation, and fever, which prevent its use in either human or veterinary vaccines. The side effects are primarily due to the host's reactions to the mycobacterial component of CFA. Incomplete Freund's adjuvant (IFA) is similar to CFA without the bacterial component. While not approved for use in the United States, IFA has been useful for several types of vaccines in other countries. IFA has been used successfully in humans with influenza and polio vaccines and with several animal vaccines including rabies, canine distemper, and foot-and-mouth disease. Experiments have shown, however, that both the oil and emulsifier used in IFA can cause tumors in mice, indicating that an alternative adjuvant would be a better choice for human use.

Muramyl dipeptide (MDP) represents the minimal unit of the mycobacterial cell wall complex that generates the adjuvant activity observed with CFA. Ellouz *et al.*, *Biochem. Biophys. Res. Comm.*, 1974, *59*, 1317. Many synthetic analogs of MDP have been generated that exhibit a wide range of adjuvant potency and side effects. Chedid *et al.*, *Prog. Allergy*, 1978, *25*, 63. Three analogs of MDP -- threonyl derivatives of MDP (Byars *et al.*, *Vaccine*, 1987, *5*, 223); n-butyl derivatives of MDP (Chedid *et al.*, *Infect. Immun.*, 1982, *35*, 417); and lipophilic derivatives of muramyl tripeptide (Gisler *et al.*, *Immunomodulations of Microbial Products and Related Synthetic Compounds*, Y. Yamamura and S. Kotani, Eds., Excerpta Medica, Amsterdam, p. 167) -- have been shown to stimulate humoral and cell-mediated immunity and exhibit low levels of toxicity. Another derivative of MDP, N-acetylmuramyl-L-alanyl-D-isoglutaminyl-L-alanine-2-[1,2-dipalmitoyl-sn-glycero-3-3(hydroxyphosphoryloxy)]ethylamide (MTP-PE) is lipophilic. MTP-PE has phospholipid tails that allow association of the hydrophobic portion of the molecule with a lipid environment while the muramyl peptide portion associates with the aqueous environment. Thus, MTP-PE itself can act as an emulsifying agent to generate stable oil in water emulsions.

Levamisole and isoprinosine are other synthetic adjuvants that increase host immunity. Levamisole is the levo isomer of tetramisole and potentiates humoral and cellular immunity through a T cell-dependent mechanism. Isoprinosine, a complex containing inosine, the purine precursor of adenosine and guanosine, promotes T cell mitogenesis. Tuftsin, a 4 amino acid peptide (Thr-Lys-Pro-Arg) homologous to a sequence in the immunoglobulin (Ig) heavy chain, primarily stimulates macrophages.

Microparticles prepared from the biodegradable and biocompatible polymers, known as the poly(lactide-co-glycolides) (PLG), have been demonstrated to be effective adjuvants for a number of antigens. In addition, PLG microparticles can control the rate of release of entrapped antigens and, thus, offer potential for single-dose vaccines. Moreover, administration of biodegradable polymers with entrapped antigens have been demonstrated in a range of animal models to induce potent immune responses. O'Hagan *et al.*, *Advanced Drug Deliv. Rev.*, 1998, *32*, 225-246 and Singh *et al.*, *Advanced Drug Deliv. Rev.*, 1998, *34*, 285-304, the disclosures of which are incorporated herein by reference in their entirety.

An emulsion comprising squalene, sorbitan trioleate (Span85™), and polysorbate 80 (Tween 80™) microfluidized to provide uniformly sized microdroplets, i.e. MF59, has also been shown to induce potent immune responses. MF59 formulations have been shown to induce antibody titers 5->100 times greater than those obtained with aluminum salt adjuvants. MF59 has been demonstrated to enhance the immune response to antigens from numerous sources including, for example, herpes simplex virus (HSV), human immunodeficiency virus (HIV), influenza virus, hepatitis C virus (HCV), cytomegalovirus (CMV), hepatitis B virus (HBV), human papillomavirus (HPV), and malaria. Ott *et al.*, *Vaccine Design: The Subunit And Adjuvant Approach*, 1995, M.F. Powell and M.J. Newman, Eds., Plenum Press, New York, p. 277-296; Singh *et al.*, *Vaccine*, 1998, *16*, 1822-1827; Ott *et al.*, *Vaccine*, 1995, *13*, 1557-1562; O'Hagan *et al.*, *Mol. Medicine Today*, 1997, February, 69-75; and Traquina *et al.*, *J. Infect. Dis.*, 1996, *174*, 1168-75, the disclosures of which are incorporated herein by reference in their entirety. MF59 adjuvant improves the immunogenicity of subunit antigens while maintaining the safety and tolerability profile of alum adjuvant. Van Nest *et al.*, *Vaccines 92*, 1992, Cold Spring Harbor Laboratory Press, 57-62 and Valensi *et al.*, *J. Immunol.*, 1994, *153*, 4029-39, the disclosures of which are

incorporated herein by reference in their entirety. MF59 is further described in co-pending U.S. application Serial No. 08/434,512, filed May 4, 1995, which is assigned to the assignee of the present invention, the disclosure of which is incorporated herein by reference in its entirety. In animal studies, MF59 has not been found to be genotoxic, teratogenic, nor does it cause sensitization. The mechanism of action of MF59 appears to be dependent upon the generation of a strong CD4+ T cell, i.e., a Th2 cell response. MF59 adjuvants, however, elicit little, if any, Th1 responses, or cytotoxic T lymphocyte (CTL) responses.

Oligonucleotides comprising CpG motifs mixed with antigens have been demonstrated to induce strong Th1 immune responses. Roman *et al.*, *Nat. Med.*, 1997, *3*, 849-854; Weiner *et al.*, *Proc. Natl. Acad. Sci. USA*, 1997, *94*, 10833-10837; Davis *et al.*, *J. Immunol.*, 1998, *160*, 870-876; Chu *et al.*, *J. Exp. Med.*, 1997, *186*, 1623-1631; Lipford *et al.*, *Eur. J. Immunol.*, 1997, *27*, 2340-2344; and Moldoveanu *et al.*, *Vaccine*, 1988, *16*, 1216-1224, the disclosures of which are incorporated herein by reference in their entirety. Unmethylated CpG dinucleotides are relatively common in bacterial DNA, but are underrepresented and methylated in vertebrate DNA. Bird, *Trends Genet.*, 1987, *3*, 342-347. Bacterial DNA or synthetic oligonucleotides containing unmethylated CpG motifs are also known to induce immune responses including, for example, B cell proliferation, interleukin-6 and immunoglobulin secretion, and apoptosis resistance. Krieg *et al.*, *Nature*, 1995, *374*, 546-549; Klinman *et al.*, *Proc. Natl. Acad. Sci. USA*, 1996, *93*, 2879-2883; Ballas *et al.*, *J. Immunol.*, 1996, *157*, 1840-1845; Cowdery *et al.*, *J. Immunol.*, 1996, *156*, 4570-4575; Halpern *et al.*, *Cell. Immunol.*, 1996, *167*, 72-78; Yamamoto *et al.*, *Jpn. J. Cancer Res.*, 1988, *79*, 866-873; Stacey *et al.*, *J. Immunol.*, 1996, *157*, 2116-2122; Messina *et al.*, *J. Immunol.*, 1991, *147*, 1759-1764; Yi *et al.*, *J. Immunol.*, 1996, *157*, 4918-4925; Yi *et al.*, *J. Immunol.*, 1996, *157*, 5394-5402; Yi *et al.*, *J. Immunol.*, 1998, *160*, 4755-4761; and Yi *et al.*, *J. Immunol.*, 1998, *160*, 5898-5906; PCT Publication WO 96/02555; PCT Publication WO 98/16247; PCT Publication WO 98/18810; PCT Publication WO 98/40100; PCT Publication WO 98/55495; PCT Publication WO 98/37919; and PCT Publication WO 98/52581, the disclosures of which are incorporated herein by reference in their entirety.

Monophosphoryl lipid A (MPL) is known to those skilled in the art to induce a Th1 lymphocyte response. Ulrich *et al.*, Monophosphoryl Lipid A as an Adjuvant in

Vaccine Design: The Subunit and Adjuvant Approach, Powell and Newman, Eds., 1995, Plenum Press, New York, p.495-523.

An adjuvant which results in the increase of a Th1 cell response which can be used for prophylactic and therapeutic treatment is, thus, still desired. Such a response would be helpful in treatment of, for example, viral infections as well as for immunizing individuals susceptible to viral infections.

SUMMARY OF THE INVENTION

The present invention is directed to adjuvant compositions which comprise an oligonucleotide comprising at least one CpG motif, and an oil droplet emulsion. The adjuvant composition can also comprise an optional component which results in a positively charged emulsion. The oil droplet emulsion preferably comprises a metabolizable oil and an emulsifying agent which are preferably present in the form of an oil-in-water emulsion having oil droplets substantially all of which are less than 1 micron in diameter. Preferably, the composition exists in the absence of any polyoxypropylene-polyoxyethylene block copolymer. The oil is preferably an animal oil, an unsaturated hydrocarbon, a terpenoid such as, for example, squalene, or a vegetable oil. The composition preferably comprises 0.5 to 20 % by volume of the oil in an aqueous medium. The emulsifying agent preferably comprises a non-ionic detergent such as a polyoxyethylene sorbitan mono-, di-, or triester or a sorbitan mono-, di-, or triether. Preferably, the composition comprises about 0.01 to about 0.5 % by weight of the emulsifying agent. The oligonucleotide preferably comprises at least one phosphorothioate bond or peptide nucleic acid bond. In preferred embodiments of the invention, the oligonucleotide comprises a nucleotide sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NO:27, and SEQ ID NO:28. In other preferred embodiments of the invention, the oligonucleotide comprises a CpG motif flanked by two purines immediately 5' to the motif and two pyrimidines

immediately 3' to the motif. In other preferred embodiments of the invention, the oligonucleotide comprises a nucleotide sequence selected from the group consisting of SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NO:27, and SEQ ID NO:28. Most preferred is SEQ ID NO:28. In some preferred embodiments of the invention, the adjuvant composition further comprises a separate immunostimulating agent which is preferably selected from the group consisting of alum, a bacterial cell wall component, and muramyl peptide. The adjuvant composition can be in the form of a microparticle.

The present invention is also directed to immunogenic compositions comprising an immunostimulating amount of an antigenic substance, and an immunostimulating amount of an adjuvant composition described herein. Preferably, the antigenic substance is selected from the group consisting of a protein, protein-polysaccharide, protein-lipopolysaccharide, polysaccharide, and lipopolysaccharide. In some embodiments of the invention, the immunogenic composition comprises a CpG oligonucleotide in combination with an antigenic substance adsorbed to poly(lactide-co-glycolide) microparticles. The adsorbed antigenic substance is preferably a recombinant protein. In preferred embodiments of the invention, the antigenic substance is from a virus such as, for example, hepatitis C virus (HCV), hepatitis B virus (HBV), herpes simplex virus (HSV), human immunodeficiency virus (HIV), cytomegalovirus (CMV), influenza virus (flu), and rabies virus. Preferably, the antigenic substance is selected from the group consisting of HSV glycoprotein gD, HIV glycoprotein gp120, and HIV p55 gag. In other preferred embodiments of the invention, the antigenic substance is from a bacterium such as, for example, cholera, diphtheria, tetanus, pertussis, *Neisseria meningitidis*, *Neisseria gonorrhoeae*, *Helicobacter pylori*, and *Haemophilus influenza*. In other preferred embodiments of the invention, the antigenic substance is from a parasite such as, for example, a malaria parasite.

The present invention is also directed to methods of stimulating an immune response in a host animal comprising administering to the animal an immunogenic composition described herein in an amount effective to induce an immune response. The host animal is preferably a mammal, more preferably a human.

The present invention is also directed to methods of immunizing a host animal against a viral, bacterial, or parasitic infection comprising administering to the animal an immunogenic composition described herein in an amount effective to induce a protective response. The host animal is preferably a mammal, more preferably a human.

The present invention is also directed to methods of increasing a Th1 immune response in a host animal comprising administering to the animal an immunogenic composition described herein in an amount effective to induce a Th1 immune response. The host animal is preferably a mammal, more preferably a human.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a bar graph showing typical results of immunoglobulin isotypes generated by preferred immunogenic compositions comprising PLG microparticles according to the invention.

Figure 2 is a bar graph showing typical results of immunoglobulin isotypes generated by preferred immunogenic compositions comprising MF59 adjuvant according to the invention.

Figure 3 is a chart showing representative results of serum anti-p55 IgG titer upon immunization with a preferred emulsion adjuvant.

Figure 4 is a chart showing representative results of lysis of targets by CTL upon immunization with a preferred emulsion adjuvant.

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

The present invention is based upon the surprising discovery that an adjuvant containing a combination of a CpG oligonucleotide and a metabolizable oil or biodegradable polymer increases Th1 lymphocyte responses, whereas other known Th1-inducing adjuvants fail to increase Th1 lymphocyte responses.

The practice of the present invention will employ, unless otherwise indicated, conventional methods of virology, immunology, microbiology, molecular biology and recombinant DNA techniques within the skill of the art. Such techniques are explained fully in the literature. See, e.g., Sambrook, et al., *Molecular Cloning: A Laboratory Manual* (2nd

Edition, 1989); *DNA Cloning: A Practical Approach*, Vols. I & II (D. Glover, ed.); *Methods In Enzymology* (S. Colowick and N. Kaplan eds., Academic Press, Inc.); *Fundamental Virology*, 2nd Edition, Vols. I & II (B.N. Fields and D.M. Knipe, eds.), *Remington's Pharmaceutical Sciences*, 18th Edition (Easton, Pennsylvania: Mack Publishing Company, 1990); *Handbook of Experimental Immunology*, Vols. I-IV (D.M. Weir and C.C. Blackwell, eds., 1986, Blackwell Scientific Publications); *Handbook of Surface and Colloidal Chemistry* (Birdi, K.S., ed, CRC Press, 1997) and *Seymour/Carrahers Polymer Chemistry* (4th edition, Marcel Dekker Inc., 1996).

As used herein, the phrase "nucleic acid" refers to DNA, RNA, or chimeras formed therefrom.

As used herein, the phrase "oligonucleotide comprising at least one CpG motif" refers to a polynucleotide comprising at least one CpG dinucleotide. Oligonucleotides comprising at least one CpG motif can comprise multiple CpG motifs. These oligonucleotide are also known in the art as "CpG oligonucleotides" in the art. As used herein, the phrase "CpG motif" refers to a dinucleotide portion of an oligonucleotide which comprises a cytosine nucleotide followed by a guanosine nucleotide. 5-methylcytosine can also be used in place of cytosine.

As used herein, the phrase "oil droplet emulsion" refers to an emulsion comprising a metabolizable oil and an emulsifying agent.

As used herein, the phrase "PLG microparticle" refers to a microparticle formulated from poly(lactide-co-glycolides).

As used herein, the term "about" means ± about 10% of the value it modifies.

According to the present invention, compositions and methods are provided which prophylactically and/or therapeutically immunize or treat a host animal against viral, fungal, mycoplasma, bacterial, or protozoan infections, as well as to tumors. The methods of the present invention are useful for conferring prophylactic and/or therapeutic immunity to a mammal, preferably a human. The methods of the present invention can also be practiced on mammals, other than humans, for biomedical research.

In one embodiment of the present invention, an oligonucleotide comprising at least one CpG motif is combined with an oil droplet emulsion to form an adjuvant. The oil

droplet emulsion preferably comprises a metabolizable oil and an emulsifying agent, wherein the oil and the emulsifying agent are present in the form of an oil-in-water emulsion having oil droplets substantially all of which are less than one micron in diameter. Such droplets show a surprising superiority over adjuvant compositions containing oil and emulsifying agents in which the oil droplets are significantly larger than those provided by the present invention. In preferred embodiments, the emulsion is positively charged as a result of a cationic detergent being used as the emulsifying agent or, alternatively, contains a cationic detergent separate from the emulsifying agent.

Although individual components of the adjuvant compositions of the present invention are known, such compositions have not been combined in the same manner. Accordingly, the individual components, although described below both generally and in some detail for preferred embodiments, are well known in the art, and the terms used herein, such as metabolizable oil, emulsifying agent, immunostimulating agent, muramyl peptide, and lipophilic muramyl peptide, are sufficiently well known to describe these compounds to one skilled in the art without further description.

One component of these compositions is a metabolizable, non-toxic oil, preferably one of about 6 to about 30 carbon atoms including, but not limited to, alkanes, alkenes, alkynes, and their corresponding acids and alcohols, the ethers and esters thereof, and mixtures thereof. The oil can be any vegetable oil, fish oil, animal oil or synthetically prepared oil which can be metabolized by the body of the host animal to which the adjuvant will be administered and which is not toxic to the subject. The host animal is typically a mammal, and preferably a human. Mineral oil and similar toxic petroleum distillate oils are expressly excluded from this invention.

The oil component of this invention can also be any long chain alkane, alkene or alkyne, or an acid or alcohol derivative thereof either as the free acid, its salt or an ester such as a mono-, or di- or triester, such as the triglycerides and esters of 1,2-propanediol or similar poly-hydroxy alcohols. Alcohols can be acylated employing amino- or poly-functional acid, for example acetic acid, propanoic acid, citric acid or the like. Ethers derived from long chain alcohols which are oils and meet the other criteria set forth herein can also be used.

The individual alkane, alkene or alkyne moiety and its acid or alcohol derivatives will generally have about 6 to about 30 carbon atoms. The moiety can have a straight or branched chain structure. It can be fully saturated or have one or more double or triple bonds. Where mono or poly ester- or ether-based oils are employed, the limitation of about 6 to about 30 carbons applies to the individual fatty acid or fatty alcohol moieties, not the total carbon count.

Any metabolizable oil, particularly from an animal, fish or vegetable source, can be used herein. It is essential that the oil be metabolized by the host to which it is administered, otherwise the oil component can cause abscesses, granulomas or even carcinomas, or (when used in veterinary practice) can make the meat of vaccinated birds and animals unacceptable for human consumption due to the deleterious effect the unmetabolized oil can have on the consumer.

Exemplary sources for vegetable oils include nuts, seeds and grains. Peanut oil, soybean oil, coconut oil, and olive oil, the most commonly available, exemplify the nut oils. Seed oils include safflower oil, cottonseed oil, sunflower seed oil, sesame seed oil and the like. In the grain group, corn oil is the most readily available, but the oil of other cereal grains such as wheat, oats, rye, rice, teff, triticale and the like can also be used.

The technology for obtaining vegetable oils is well developed and well known. The compositions of these and other similar oils can be found in, for example, the Merck Index, and source materials on foods, nutrition and food technology.

The 6-10 carbon fatty acid esters of glycerol and 1,2-propanediol, while not occurring naturally in seed oils, can be prepared by hydrolysis, separation and esterification of the appropriate materials starting from the nut and seed oils. These products are commercially available under the name NEOBEE® from PVO International, Inc., Chemical Specialties Division, 416 Division Street, Boongon, NJ, and others.

Oils from any animal source can also be employed in the adjuvants and immunogenic compositions of this invention. Animal oils and fats are usually solids at physiological temperatures due to the fact that they exist as triglycerides and have a higher degree of saturation than oils from fish or vegetables. However, fatty acids are obtainable from animal fats by partial or complete triglyceride saponification which provides the free

fatty acids. Fats and oils from mammalian milk are metabolizable and can therefore be used in the practice of this invention. The procedures for separation, purification, saponification and other means necessary for obtaining pure oils from animal sources are well known in the art.

Most fish contain metabolizable oils which can be readily recovered. For example, cod liver oil, shark liver oils, and whale oil such as spermaceti exemplify several of the fish oils which can be used herein. A number of branched chain oils are synthesized biochemically in 5-carbon isoprene units and are generally referred to as terpenoids. Shark liver oil contains a branched, unsaturated terpenoid known as squalene, 2,6,10,15,19,23-hexamethyl-2,6,10,14,18,22-tetracosahexaene which is particularly preferred herein. Squalane, the saturated analog to squalene, is also a particularly preferred oil. Fish oils, including squalene and squalane, are readily available from commercial sources or can be obtained by methods known in the art.

The oil component of these adjuvants and immunogenic compositions will be present in an amount from about 0.5% to about 20% by volume but preferably no more than about 15%, especially in an amount of about 1% to about 12%. It is most preferred to use from about 1% to about 4% oil.

The aqueous portion of these adjuvant compositions is preferably buffered saline or, more preferably, unadulterated water. Because these compositions are intended for parenteral administration, it is preferable to make up final buffered solutions used as immunogenic compositions so that the tonicity, i.e., osmolality, is essentially the same as normal physiological fluids in order to prevent post-administration swelling or rapid absorption of the composition because of differential ion concentrations between the composition and physiological fluids. It is also preferable to buffer the saline in order to maintain pH compatible with normal physiological conditions. Also, in certain instances, it can be necessary to maintain the pH at a particular level in order to ensure the stability of certain composition components such as the glycopeptides.

Any physiologically acceptable buffer can be used herein, but phosphate buffers are preferred. Other acceptable buffers such acetate, tris, bicarbonate, carbonate, or

the like can be used as substitutes for phosphate buffers. The pH of the aqueous component will preferably be between about 6.0-8.0.

When the adjuvant is initially prepared, however, unadulterated water is preferred as the aqueous component of the emulsion. Increasing the salt concentration makes it more difficult to achieve the desired small droplet size. When the final immunogenic compositions is prepared from the adjuvant, the antigenic material can be added in a buffer at an appropriate osmolality to provide the desired immunogenic composition.

The quantity of the aqueous component employed in these compositions will be that amount necessary to bring the value of the composition to unity. That is, a quantity of aqueous component sufficient to make 100% will be mixed, with the other components listed above, in order to bring the compositions to volume.

A substantial number of emulsifying and suspending agents are generally used in the pharmaceutical sciences. These include naturally derived materials such as gums from trees, vegetable protein, sugar-based polymers such as alginates and cellulose, and the like. Certain oxypolymers or polymers having a hydroxide or other hydrophilic substituent on the carbon backbone have surfactant activity, for example, povidone, polyvinyl alcohol, and glycol ether-based mono- and poly-functional compounds. Long chain fatty-acid-derived compounds form a third substantial group of emulsifying and suspending agents which could be used in this invention. Any of the foregoing surfactants are useful so long as they are non-toxic.

Specific examples of suitable emulsifying agents (also referred to as surfactants or detergents) which can be used in accordance with the present invention include the following:

1. Water-soluble soaps, such as the sodium, potassium, ammonium and alkanol-animonium salts of higher fatty acids (C_{10}-C_{22}), and, particularly sodium and potassium tallow and coconut soaps.

2. Anionic synthetic non-soap detergents, which can be represented by the water-soluble salts of organic sulfuric acid reaction products having in their molecular structure an alkyl radical containing from about 8 to about 22 carbon atoms and a radical selected from the group consisting of sulfonic acid and sulfuric acid ester radicals. Examples

of these are the sodium or potassium alkyl sulfates, derived from tallow or coconut oil; sodium or potassium alkyl benzene sulfonates; sodium alkyl glyceryl ether sulfonates; sodium coconut oil fatty acid monoglyceride sulfonates and sulfates; sodium or potassium salts of sulfuric acid asters of the reaction product of one mole of a higher fatty alcohol and about 1 to about 6 moles of ethylene oxide; sodium or potassium alkyl phenol ethylene oxide ether sulfonates, with 1 to about10 units of ethylene oxide per molecule and in which the alkyl radicals contain from about 8 to about 12 carbon atoms; the reaction product of fatty acids esterified with isethionic acid and neutralized with sodium hydroxide; sodium or potassium salts of fatty acid amide of a methyl tauride; and sodium and potassium salts of SO_3- sulfonated C_{10}-C_{24} α-olefins.

3. Nonionic synthetic detergents made by the condensation of alkylene oxide groups with an organic hydrophobic compound. Typical hydrophobic groups include condensation products of propylene oxide with propylene glycol, alkyl phenols, condensation product of propylene oxide and ethylene diamine, aliphatic alcohols having about 8 to about 22 carbon atoms, and amides of fatty acids.

4. Nonionic detergents, such as amine oxides, phosphine oxides and sulfoxides, having semipolar characteristics. Specific examples of long chain tertiary amine oxides include dimethyldodecylamine oxide and bis-(2-hydroxyethyl) dodecylamine. Specific examples of phosphine oxides are found in U.S. Patent No. 3,304,263 which issued February 14, 1967, and include dimethyldodecylphosphine oxide and dimethyl-(2-hydroxydodecyl) phosphine oxide.

5. Long chain sulfoxides, including those corresponding to the formula R^1-SO-R^2 wherein R^1 and R^2 are substituted or unsubstituted alkyl radicals, the former containing from about 10 to about 28 carbon atoms, whereas R^2 contains from 1 to about 3 carbon atoms. Specific examples of these sulfoxides include dodecylmethyl sulfoxide and 3-hydroxy tridecyl methyl sulfoxide.

6. Ampholytic synthetic detergents, such as sodium 3-dodecylamino-propionate and sodium 3-dodecylaminopropane sulfonate.

7. Zwitterionic synthetic detergents, such as 3-(N,N-dimethyl-N-hexadecylammonio) propane-l-sulfonate and 3-(N,N-dimethyl-N-hexadecylammonio)-2-hydroxy propane-l-sulfonate.

Additionally, all of the following types of emulsifying agents can be used in a composition of the present invention: (a) soaps (*i.e.*, alkali salts) of fatty acids, rosin acids, and tall oil; (b) alkyl arene sulfonates; (c) alkyl sulfates, including surfactants with both branched-chain and straight chain hydrophobic groups, as wall as primary and secondary sulfate groups; (d) sulfates and sulfonates containing an intermediate linkage between the hydrophobic and hydrophilic groups, such as the fatty acylated methyl taurides and the sulfated fatty monoglycerides; (e) long-chain acid esters of polyethylene glycol, especially the tall oil esters; (f) polyethylene glycol ethers of alkylphenols; (g) polyethylene glycol ethers of long-chain alcohols and mercaptans; and (h) fatty acyl diethanol amides. Since surfactants can be classified in more than one manner, a number of classes of surfactants set forth in this paragraph overlap with previously described surfactant classes.

There are a number oil emulsifying agents specifically designed for and commonly used in biological situations. For example, a number of biological detergents (surfactants) are listed as such by Sigma Chemical Company on page 310-316 of its 1987 Catalog of Biochemical and Organic Compounds. Such surfactants are divided into four basic types: anionic, cationic, zwitterionic, and nonionic. Examples of anionic detergents include, but are not limited to, alginic acid, caprylic acid, cholic acid, 1-decanesulfonic acid, deoxycholic acid, 1-dodecanesulfonic acid, N-lauroylsarcosine, and taurocholic acid, and the like. Cationic detergents include, but are not limited to, cetrimide (hexadecyltrimethylammonium bromide – CTAB), benzalkonium chloride, dimethyl dioctodecyl ammonium (DDA) bromide, DOTAP, dodecyltrimethylammonium bromide, benzyldimethylhexadecyl ammonium chloride, cetylpyridinium chloride, methylbenzethonium chloride, and 4-picoline dodecyl sulfate, and the like. Examples of zwitterionic detergents include, but are not limited to, 3-[(3-cholamidopropyl)-dimethylammonio]-l-propanesulfonate (commonly abbreviated CHAPS), 3-[(cholamidopropyl)-dimethylammonio]-2-hydroxy-l-propanesulfonate (generally abbreviated CHAPSO) N-dodecyl-N,N-dimethyl-3-ammonio-l-propanesulfonate, and lyso-α-phosphatidylcholine, and the like. Examples of nonionic

detergents include, but are not limited to, decanoyl-N-methylglucamide, diethylene glycol monopentyl ether, n-dodecyl β-D-glucopyranoside, ethylene oxide condensates of fatty alcohols (e.g., sold under the trade name Lubrol), polyoxyethylene ethers of fatty acids (particularly C_{12}-C_{20} fatty acids), polyoxyethylene sorbitan fatty acid ethers (e.g., sold under the trade name Tween), and sorbitan fatty acid ethers (e.g., sold under the trade name Span), and the like. The optional component of the adjuvant compositions which results in a positively charged emulsion can be, for example, any of the cationic detergents described above. Alternatively, the cationic detergents described above can be used along with any of the oil droplet emulsions described above in order to render the emulsion positively charged.

A particularly useful group of surfactants are the sorbitan-based non-ionic surfactants. These surfactants are prepared by dehydration of sorbitol to give 1,4-sorbitan which is then reacted with one or more equivalents of a fatty acid. The fatty-acid substituted moiety can be further reacted with ethylene oxide to give a second group of surfactants.

The fatty-acid-substituted sorbitan surfactants are made by reacting 1,4-sorbitan with a fatty acid such as lauric acid, palmitic acid, stearic acid, oleic acid, or a similar long chain fatty acid to give the 1,4-sorbitan mono-ester, l,g-sorbitan sesquiester or 1,4-sorbitan triester. The common names for these surfactants include, for example, sorbitan monolaurate, sorbitan monopalmitate, sorbitan monoestearate, sorbitan monooleate, sorbitan sesquioleate, and sorbitan trioleate. These surfactants are commercially available under the name SPAN® or ARLACEL®, usually with a letter or number designation which distinguishes between the various mono-, di- and triester substituted sorbitans.

SPAN® and ARLACEL® surfactants are hydrophilic and are generally soluble or dispersible in oil. They are also soluble in most organic solvents. In water they are generally insoluble but dispersible. Generally these surfactants will have a hydrophilic-lipophilic balance (HLB) number between 1.8 to 8.6. Such surfactants can be readily made by means known in the art or are commercially available from, for example, ICI America's Inc., Wilmington, DE under the registered mark ATLAS®.

A related group of surfactants comprises polyoxyethylene sorbitan monoesters and polyoxyethylene sorbitan triesters. These materials are prepared by addition of ethylene oxide to a 1,4-sorbitan monoester or triester. The addition of polyoxyethylene converts the

lipophilic sorbitan mono- or triester surfactant to a hydrophilic surfactant generally soluble oil dispersible in water and soluble to varying degrees in organic liquids.

These materials, commercially available under the mark TWEEN® are useful for preparing oil-in-water emulsions and dispersions or for the solubilization of oils and making anhydrous ointments water-soluble or washable. The TWEEN® surfactants can be combined with a related sorbitan monoester or triester surfactants to promote emulsion stability. TWEEN® surfactants generally have a HLB value falling between 9.6 to 16.7.

A third group of non ionic surfactants which could be used alone or in combination with SPAN®, ARLACEL®, and TWEEN® surfactants are the polyoxyethylene fatty acids made by the reaction of ethylene oxide with a long-chain fatty acid. The most commonly available surfactant of this type is solid under the name MYRJ® and is a polyoxyethylene derivative of stearic acid. MYRJ® surfactants are hydrophilic and soluble or dispersible in water like TWEEN® surfactants. The MYRJ® surfactants can be blended with TWEEN® surfactants, or with TWEEN®/SPAN® or ARLACEL® surfactant mixtures for use in forming emulsions. MYRJ® surfactants can be made by methods known in the art or are available commercially from ICI America's Inc.

A fourth group of polyoxyethylene based nonionic surfactants are the polyoxyethylene fatty acid ethers derived from lauryl, acetyl, stearyl and oleyl alcohols. These materials are prepared as above by addition of ethylene oxide to a fatty alcohol. The commercial name for these surfactants is BRIJ®. BRIJ® surfactants can be hydrophilic or lipophilic depending on the size of the polyoxyethylene moiety in the surfactant. While the preparation of these compounds is available from the art, they are also readily available from such commercial sources as ICI America's Inc.

Other non-ionic surfactants which could potentially be used in the practice of this invention are for example: polyoxyethylene, polyol fatty acid esters, polyoxyethylene ether, polyoxypropylene fatty ethers, bee's wax derivatives containing polyoxyethylene, polyoxyethylene lanolin derivative, polyoxyethylen fatty glyceride, glycerol fatty acid esters or other polyoxyethylene acid alcohol or ether derivatives of long-chain fatty acids of 12-22 carbon atoms.

As the adjuvant and the immunogenic compositions of this invention are intended to be multi-phase systems, it is preferable to choose an emulsion-forming non-ionic surfactant which has an HLB value in the range of about 7 to about16. This value can be obtained through the use of a single non-ionic surfactant such as a TWEEN® surfactant or can be achieved by the use of a blend of surfactants such as with a sorbitan mono, di- or triester based surfactant; a sorbitan ester polyoxyethylene fatty acid; a sorbitan ester in combination with a polyoxyethylene lanolin derived surfactant; a sorbitan ester surfactant in combination with a high HLB polyoxyethylene fatty ether surfactant; or a polyethylene fatty ether surfactant or polyoxyethylene sorbitan fatty acid.

It is more preferred to use a single nonionic surfactant, most particularly a TWEEN® surfactant, as the emulsion stabilizing non-ionic surfactant in the practice of this invention. The surfactant named TWEEN® 80, otherwise known as polysorbate 80 for polyoxyethlyene 20 sorbitan monooleate, is the most preferred of the foregoing surfactants.

Sufficient droplet size reduction can usually be effected by having the surfactant present in an amount of 0.02% to 2.5% by weight (w/w). An amount of 0.05% to 1% is preferred with 0.01 to 0.5% being especially preferred.

The manner in which the droplet size of the invention is reached is not important to the practice of the present invention. One manner in which submicron oil droplets can be obtained is by use of a commercial emulsifiers, such as model number 110Y available from Microfluidics, Newton, MA. Examples of other commercial emulsifiers include Gaulin Model 30CD (Gaulin, Inc., Everett, MA) and Raimie Minilab Type 8.30H (Miro Atomizer Food and Dairy, Inc., Hudson, WI). These emulsifiers operated by the principle of high shear forces developed by forcing fluids through small apertures under high pressure. When the model 110Y is operated at 5,000-30,000 psi, oil droplets having diameters of 100 - 750 nm are provided.

The size of the oil droplets can be varied by changing the ratio of detergent to oil (increasing the ratio decreases droplet size, operating pressure (increasing operating pressure reduces droplet size), temperature (increasing temperature decreases droplet size), and adding an amphipathic immunostimulating agent (adding such agents decreases droplet size). Actual droplet size will vary with the particular detergent, oil, and immunostimulating

agent (if any) and with the particular operating conditions selected. Droplet size can be verified by use of sizing instruments, such as the commercial Sub-Micron Particle Analyzer (Model N4MD) manufactured by the Coulter Corporation, and the parameters can be varied using the guidelines set forth above until substantially all droplets are less than 1 micron in diameter, preferably less than 0.8 microns in diameter, and most preferably less than 0.5 microns in diameter. By substantially all is meant at least about 80% (by number), preferably at least about 90%, more preferably at least about 95%, and most preferably at least about 98%. The particle size distribution is typically Gaussian, so that the average diameter is smaller than the stated limits.

The present invention is practiced by preparing an oil emulsion in the absence of other components previously taught in the prior art to be used with submicron emulsions for satisfactory immunogenicity, namely polyoxylropylene-polyoxyethlyne block polymers such as those described for use with adjuvants in U.S. Patent Numbers 4,772,466 and 4,770,874 and in European Patent Application 0 315 153 A2.

An adjuvant composition of the invention comprises a metabolizable oil in water and an emulsifying agent other than a POP-POE copolymer. The emulsifying agent need not have any specific immunostimulating activity, since the oil composition by itself can function as an adjuvant when the oil droplets are in the sub-micron range. However, increased immunostimulating activity can be provided by including any of the known immunostimulating agents in the composition. These immunostimulating agents can either be separate from the emulsifying agent and the oil or the immunostimulating agent and the emulsifying agent can be one and the same molecule. Examples of the former situation include metabolizable oils mixed with killed mycobacteria, such as *Mycobacterium tuberculosis*, and subcellular components thereof. Additional immunostimulating substances include the muramyl peptides that are components of the cell walls of such bacteria, and include derivatives thereof. Examples of the joint emulsifying agent/immunostimulating agent are the lipophilic muramyl peptides described in Sanchez-Pescador *et al.*, *J. Immunol.*, **1988**, ***141***, 1720-1727, the disclosure of which is incorporated herein by reference in its entirety. These materials comprise the basic N-acetylmuramyl peptide (a hydrophilic moiety) that acts is an immunostimulating group, but also include a lipophilic moiety that provides surface-

active characteristics to the resulting compound. Such compounds, as well as other types of amphipathic immunostimulating substances, act as both immunostimulating agents and emulsifying agents and are preferred in the practice of the present invention. In addition, it is also possible to practice the present invention by using a amphipathic immunostimulating substance in combination with a second immunostimulating substance that is not amphipathic. An example would be use of a lipophilic muramyl peptide in combination with an essentially unsubstituted (i.e., essentially hydrophilic) muramyl dipeptide.

A preferred oil droplet emulsion is MF59. MF59 can be made according to the procedures described in, for example, Ott *et al.*, *Vaccine Design: The Subunit And Adjuvant Approach*, 1995, M.F. Powell and M.J. Newman, Eds., Plenum Press, New York, p. 277-296; Singh *et al.*, *Vaccine*, 1998, *16*, 1822-1827; Ott *et al.*, *Vaccine*, 1995, *13*, 1557-1562; and Valensi *et al.*, *J. Immunol.*, 1994, *153*, 4029-39, the disclosures of which are incorporated herein by reference in their entirety.

Other oil droplet emulsions include, for example, SAF, containing 10% Squalane, 0.4% Tween 80, 5% pluronic-blocked polymer L121, and thr-MDP either microfluidized into a submicron emulsion or vortexed to generate a larger particle size emulsion, and Ribi® adjuvant system (RAS), (Ribi Immunochem, Hamilton, MT) containing 2% Squalene, 0.2% Tween 80, and one or more bacterial cell wall components from the group consisting of monophosphorylipid A (MPL), trehalose dimycolate (TDM), and cell wall skeleton (CWS), preferably MPL + CWS (DetoxJ) (for a further discussion of suitable submicron oil-in-water emulsions for use herein, see commonly owned, patent application no. 09/015,736, filed on January 29, 1998).

The second component of the compositions of the present invention is an oligonucleotide which comprises at least one CpG motif. As used herein, the phrase "CpG motif" refers to a dinucleotide portion of an oligonucleotide which comprises a cytosine nucleotide followed by a guanosine nucleotide. Such oligonucleotides can be prepared using conventional oligonucleotide synthesis well known to the skilled artisan. Preferably, the oligonucleotides of the invention comprise a modified backbone, such as a phosphorothioate or peptide nucleic acid, so as to confer nuclease resistance to the oligonucleotide. Modified backbones are well known to those skilled in the art. Preferred peptide nucleic acids are

described in detail in U.S. Patent Numbers 5,821,060, 5,789,573, 5,736,392, and 5,721,102, Japanese Patent No. 10231290, European Patent No. 839,828, and PCT Publication Numbers WO 98/42735, WO 98/42876, WO 98/36098, WO 98/27105, WO 98/20162, WO 98/16550, WO 98/15648, WO 98/04571, WO 97/41150, WO 97/39024, and WO 97/38013, the disclosures of which are incorporated herein by reference in their entirety.

The oligonucleotide preferably comprises between about 6 and about 100 nucleotides, more preferably between about 8 and about 50 nucleotides, most preferably between about 10 and about 40 nucleotides. In addition, the oligonucleotides of the invention can comprise substitutions of the sugar moieties and nitrogenous base moieties. Preferred oligonucleotides are disclosed in, for example, Krieg *et al.*, *Proc. Natl. Acad. Sci. USA*, 1998, *95*, 12631-12636, Klinman *et al.*, *Proc. Natl. Acad. Sci. USA*, 1996, *93*, 2879-2883, Weiner *et al.*, *Proc. Natl. Acad. Sci. USA*, 1997, *94*, 10833-10837, Chu *et al.*, *J. Exp. Med.*, 1997, *186*, 1623-1631, Brazolot-Millan *et al.*, *Proc. Natl. Acad. Sci. USA*, 1998, *95*, 15553-15558, Ballas *et al.*, *J. Immunol.*, 1996, *157*, 1840-1845, Cowdery *et al.*, *J. Immunol.*, 1996, *156*, 4570-4575, Halpern *et al.*, *Cell. Immunol.*, 1996, *167*, 72-78, Yamamoto *et al.*, *Jpn. J. Cancer Res.*, 1988, *79*, 866-873, Stacey *et al.*, *J. Immunol.*, 1996, *157*, 2116-2122, Messina *et al.*, *J. Immunol.*, 1991, *147*, 1759-1764, Yi *et al.*, *J. Immunol.*, 1996, *157*, 4918-4925, Yi *et al.*, *J. Immunol.*, 1996, *157*, 5394-5402, Yi *et al.*, *J. Immunol.*, 1998, *160*, 4755-4761, Roman *et al.*, *Nat. Med.*, 1997, *3*, 849-854, Davis *et al.*, *J. Immunol.*, 1998, *160*, 870-876, Lipford *et al.*, *Eur. J. Immunol.*, 1997, *27*, 2340-2344, Moldoveanu *et al.*, *Vaccine*, 1988, *16*, 1216-1224, Yi *et al.*, *J. Immunol.*, 1998, *160*, 5898-5906, PCT Publication WO 96/02555, PCT Publication WO 98/16247, PCT Publication WO 98/18810, PCT Publication WO 98/40100, PCT Publication WO 98/55495, PCT Publication WO 98/37919, and PCT Publication WO 98/52581, the disclosures of which are incorporated herein by reference in their entirety. It is to be understood that the oligonucleotides of the invention comprise at least one CpG motif but can contain a plurality of CpG motifs.

Preferred oligonucleotides comprise nucleotide sequences such as, for example, tccatgacgttcctgacgtt (SEQ ID NO:1), ataatcgacgttcaagcaag (SEQ ID NO:2), ggggtcaacgttgaggggg (SEQ ID NO:3), tctcccagcgtgcgccat (SEQ ID NO:4), gagaacgctcgaccttcgat (SEQ ID NO:5), tccatgtcgttcctgatgct (SEQ ID NO:6),

tccatgacgttcctgatgct (SEQ ID NO:7), gctagacgttagcgt (SEQ ID NO:8), atcgactctcgagcgttctc (SEQ ID NO:9), gaaccttccatgctgttccg (SEQ ID NO:10), gctagatgttagcgt (SEQ ID NO:11), tcaacgtt (SEQ ID NO:12), gcaacgtt (SEQ ID NO:13), tcgacgtc (SEQ ID NO:14), tcagcgct (SEQ ID NO:15), tcaacgct (SEQ ID NO:16), tcatcgat (SEQ ID NO:17), tcttcgaa (SEQ ID NO:18), tgactgtgaacgttcgagatga (SEQ ID NO:19), tgactgtgaacgttagcgatga (SEQ ID NO:20), tgactgtgaacgttagagcgga (SEQ ID NO:21), gtttgcgcaacgttgttgccat (SEQ ID NO:22), atggcaacaacgttgcgcaaac (SEQ ID NO:23), cattggaaaacgttcttcgggg (SEQ ID NO:24), ccccgaagaacgttttccaatg (SEQ ID NO:25), attgacgtcaat (SEQ ID NO:26), ctttccattgacgtcaatgggt (SEQ ID NO:27), and tccatacgttcctgacgtt (SEQ ID NO:28). In preferred embodiments of the invention, the oligonucleotide comprises a CpG motif flanked by two purines at the 5' side of the motif and two pyrimidines at the 3' side of the motif. It is to be understood, however, that any oligonucleotide comprising a CpG motif can be used in the present invention as long as the oligonucleotide induces an increase in Th1 lymphocyte stimulation when combined with the oil droplet emulsions described herein.

The present invention is also directed to immunogenic compositions comprising the compositions described above in combination with an antigenic substance. Preferably, the immunogenic composition comprises an oil droplet emulsion along with an oligonucleotide comprising at least one CpG motif. The adjuvant compositions are generally prepared from the ingredients described above prior to combining the adjuvant with the antigenic substance that will be used in the immunogenic composition. The word antigen or antigenic substance refers to any substance, including a protein or protein-polysaccharide, protein-lipopolysaccharide, polysaccharide, lipopolysaccharide, viral subunit, whole virus or whole bacteria which, when foreign to the blood stream of an animal, on gaining access to the tissue of such an animal, stimulates the formation of specific antibodies and reacts specifically *in vivo* or *in vitro* with a homologous antibody. Moreover, it stimulates the proliferation of T-lymphocytes, preferably Th1 lymphocytes, with receptors for the antigen and can react with the lymphocytes to initiate the series of responses designated cell-mediated immunity.

A hapten is within the scope of this definition of antigen. A hapten is that portion of an antigenic molecule or antigenic complex that determines it immunological specificity. Commonly, a hapten is a peptide or polysaccharide in naturally occurring

antigens. In artificial antigens it can be a low molecular weight substance such as an arsanilic acid derivative. A hapten will react specifically *in vivo* or *in vitro* with homologous antibodies or T lymphocytes. Alternative descriptors are antigenic determinant, antigenic structural grouping and haptenic grouping.

In preferred embodiments of the invention, the antigenic substance is derived from a virus such as, for example, human immunodeficiency virus (HIV), hepatitis B virus (HBV), hepatitis C virus (HCV), herpes simplex virus (HSV), cytomegalovirus (CMV), influenza virus (flu), and rabies virus. Preferably, the antigenic substance is selected from the group consisting of HSV glycoprotein gD, HIV glycoprotein gp120, and HIV p55 gag. In other preferred embodiments of the invention, the antigenic substance is derived from a bacterium such as, for example, *Helicobacter pylori, Haemophilus influenza,* cholera, diphtheria, tetanus, *Neisseria meningitidis,* and pertussis. In other preferred embodiments of the invention, the antigenic substance is from a parasite such as, for example, a malaria parasite.

Antigens can be produced by methods known in the art or can be purchased from commercial sources. Antigens within the scope of this invention include whole inactivated virus particles, isolated virus proteins and protein subunits, whole cells and bacteria, cell membrane and cell wall proteins, and the like. Some preferred antigens are described below.

Herpes simplex virus (HSV) rgD2 is a recombinant protein produced in genetically engineered Chinese hamster ovary cells. This protein has the normal anchor region truncated, resulting in a glycosylated protein secreted into tissue culture medium. The gD2 can be purified in the CHO medium to greater than 90% purity. Human immunodeficiency virus (HIV) env-2-3 is a recombinant form of the HIV enveloped protein produced in genetically engineered *Saccharomyces cerevisae.* This protein represents the entire protein region of HIV gp120 but is nonglycosylated and denatured as purified from the yeast. HIV gp120 is a fully glycosylated, secreted form of gp120 produced in CHO cells in a fashion similar to the gD2 above. Additional HSV antigens suitable for use in immunogenic compositions are described in PCT Publications W0 85/04587 and W0 88/02634, the disclosures of which are incorporated herein by reference in their entirety. Mixtures of gB and

gD antigens, which are truncated surface antigens lacking the anchor regions, are particularly preferred.

Influenza antigens suitable for use in immunogenic compositions are commercially available. Antigens that can be used in the following examples include, but are not limited to FLUOGEN® (manufactured by Parke-Davis), Duphar (manufactured by Duphar B.V.), and influenza vaccine batch A41 (manufactured by Instituto Vaccinogeno Pozzi).

Malaria antigens suitable for use in immunogenic compositions are described in U.S. patent application Serial No. 336,288, filed April 11, 1989, and in U.S. Patent No. 4,826,957, the disclosures of which are incorporated herein by reference in their entirety.

Additional HIV antigens suitable for use in immunogenic compositions are described in U.S. application serial No. 490,858, filed March 9, 1990, and published European application number 181150 (May 14, 1986), disclosures of which are incorporated herein by reference in their entirety.

Cytomegalovirus antigens suitable for use in immunogenic compositions are described in U.S. Patent No. 4,689,225, U.S. application serial number 367,363, filed June 16, 1989 and PCT Publication WO 89/07143, the disclosures of which are incorporated herein by reference in their entirety.

Hepatitis C antigens suitable for use in immunogenic compositions are described in PCT/US88/04125, published European application number 318216 (May 31, 1989), published Japanese application number 1-500565 filed November 18, 1988, Canadian application 583,561, and EPO 388,232, disclosures of which are incorporated herein by reference in their entirety. A different set of HCV antigens is described in European patent application 90/302866.0, filed March 16, 1990, and U.S. application serial number 456,637, filed December 21, 1989, and PCT/US90/01348, the disclosures of which are incorporated herein by reference in their entirety.

Immunogenic compositions of the invention can be used to immunize birds and mammals against diseases and infection, including without limitation cholera, diphtheria, tetanus, pertussis, influenza, measles, meningitis, mumps, plague, poliomyelitis, rabies, Rocky Mountain spotted fever, rubella, smallpox, typhoid, typhus, feline leukemia virus, and yellow fever.

In other embodiments of the invention, poly(lactide-co-glycolide) (PLG) microparticles to which proteins are absorbed can be used in place of the oil droplet emulsions of the present invention. These PLG microparticles can be prepared according to the procedures described in, for example, O'Hagan *et al.*, *Advanced Drug Deliv. Rev.*, 1998, *32*, 225-246 and Singh *et al.*, *Advanced Drug Deliv. Rev.*, 1998, *34*, 285-304, the disclosures of which are incorporated herein by reference in their entirety. Preferably, the protein absorbed to the PLG microparticles is a recombinant protein. The term "microparticle" as used herein, refers to a particle of about 100 nm to about 150 μm in diameter, more preferably about 200 nm to about 30 μm in diameter, and most preferably about 500 nm to about 10 μm in diameter. Preferably, the microparticle will be of a diameter that permits parenteral or mucosal administration without occluding needles and capillaries. Microparticle size is readily determined by techniques well known in the art, such as photon correlation spectroscopy, laser diffractometry and/or scanning electron microscopy. Alternatively, microparticles comprising the oil droplet emulsion can be used and are described in, for example, co-pending international application Serial No. PCT/US99/17308, filed July 29, 1999, which is incorporated herein by reference in its entirety.

The compositions of an immunogenic composition of the invention will employ an effective amount of an antigen. That is, there will be included an amount of antigen which, in combination with the adjuvant, will cause the subject to produce a specific and sufficient immunological response, preferably a Th1 lymphocyte response, so as to impart protection to the subject from the subsequent exposure to virus, bacterium, fungus, mycoplasma, or parasite immunized against.

No single dose designation can be assigned which will provide specific guidance for each and every antigen which can be employed in this invention. The effective amount of antigen will be a function of its inherent activity and purity and is empirically determined by those of ordinary skill in the art via routine experimentation. It is contemplated that the adjuvant compositions of this invention can be used in conjunction with whole cell or viral immunogenic compositions as well as with purified antigens or protein subunit or peptide immunogenic compositions prepared by recombinant DNA techniques or synthesis. Since the adjuvant compositions of the invention are stable, the antigen and emulsion can

mixed by simple shaking. Other techniques, such as passing a mixture of the adjuvant and solution or suspension of the antigen rapidly through a small opening (such as a hypodermic needle) readily provides a useful immunogenic composition.

The immunogenic compositions according to the present invention comprise about 1 nanogram to about 1000 micrograms of nucleic acid, preferably DNA such as, for example, CpG oligonucleotides. In some preferred embodiments, the immunogenic compositions contain about 10 nanograms to about 800 micrograms of nucleic acid. In some preferred embodiments, the immunogenic compositions contain about 0.1 to about 500 micrograms of nucleic acid. In some preferred embodiments, the immunogenic compositions contain about 1 to about 350 micrograms of nucleic acid. In some preferred embodiments, the immunogenic compositions contain about 25 to about 250 micrograms of nucleic acid. In some preferred embodiments, the immunogenic compositions contain about 100 micrograms nucleic acid. One skilled in the art can readily formulate an immunogenic composition comprising any desired amount of nucleic acid. The immunogenic compositions according to the present invention are provided sterile and pyrogen free. The immunogenic compositions can be conveniently administered in unit dosage form and can be prepared by any of the methods well known in the pharmaceutical art, for example, as described in *Remington's Pharmaceutical Sciences* (Mack Pub. Co., Easton, PA, 1980), the disclosure of which is incorporated herein by reference in its entirety.

The present invention is also directed to methods of stimulating an immune response in a host animal comprising administering to the animal an immunogenic composition described above in an amount effective to induce an immune response. The host animal is preferably a mammal, more preferably a human. Preferred routes of administration include, but are not limited to, intramuscular, intraperitoneal, intradermal, subcutaneous, intravenous, intraarterially, intraoccularly and oral as well as transdermally or by inhalation or suppository. Most preferred routes of administration include intramuscular, intraperitoneal, intradermal and subcutaneous injection. According to some embodiments of the present invention, the immunogenic composition is administered to a host animal using a needleless injection device, which are well known and widely available. One having ordinary skill in the

art can, following the teachings herein, use needleless injection devices to deliver immunogenic compositions to cells of an individual.

The present invention is also directed to methods of immunizing a host animal against a viral, bacterial, or parasitic infection comprising administering to the animal an immunogenic composition described above in an amount effective to induce a protective response. The host animal is preferably a mammal, more preferably a human. Preferred routes of administration are described above. While prophylactic or therapeutic treatment of the host animal can be directed to any pathogen, preferred pathogens, including, but not limited to, the viral, bacterial and parasitic pathogens described above.

The present invention is also directed to methods of increasing a Th1 immune response in a host animal comprising administering to the animal an immunogenic composition described above in an amount effective to induce a Th1 immune response. The host animal is preferably a mammal, more preferably a human. Preferred routes of administration are described above. One skilled in the art is readily familiar with Th1 lymphocytes and responses and measurements thereof.

The invention is further illustrated by way of the following examples which are intended to elucidate the invention. The foregoing examples are meant to illustrate the invention and are not to be construed to limit the invention in any way. Those skilled in the art will recognize modifications that are within the spirit and scope of the invention. All references cited herein are hereby incorporated by reference in their entirety.

EXAMPLES

Example 1: Preparation of Adjuvant Compositions

MTP-PE was provided by CIBA-GEIGY (Basel, Switzerland). Squalene and TWEEN® 80 were obtained from Sigma Chemical Co. (St. Louis, MO). CFA and IFA were obtained from Gibco (Grand Island, NY). Aluminum hydroxide (Rehsorptar) was obtained from Reheis Chemical Co. (Berkeley Heights NJ).

Preparation of oil droplet emulsions was made by a number of methods. In the first method, a mixture consisting of 4% squalene, 0.008% TWEEN® 80, 250 µg/ml MTP-PE and antigen in phosphate buffered saline (PBS) was passed through a 23 gauge needle 6 times.

This emulsion consisted of oil droplet sizes in the range of 10 microns and is termed MTP-PE-LO. The second method comprises passing the above-described mixture through a Kirkland emulsifier five times. This emulsion consists of oil droplets primarily of 1-2 microns and is termed MTP-PE-LO-KE. The Kirkland emulsifier (Kirkland Products, Walnut Creek, CA) is a small-scale version of the commercial knife-edged homogenizer (e.g., Gaulin Model 30CD and Rainnie Minilab Type 8.30H) generating about 1000 psi in the working chamber. In the third method, mixtures containing 0.3-18% squalene and 0.2-1.0 mg/ml MTP-PE with or without TWEEN®80 were passed through the Microfluidizer (Model No. 110Y Microfluidics, Newton, MA) at 5,000 - 30,000 psi. Typically, 50 ml of emulsion was mixed for 5 minutes or 100 ml for 10 minutes in the microfluidizer. The resulting emulsions consisted of oil droplets of 100 - 750 nm depending on squalene, MTP-PE, and detergent concentration and microfluidizer operating pressure and temperature. This compositions is termed MTP-PE-LO-MF.

Example 2: Preparation Of Microparticles Using CTAB

Blank microparticles were produced using CTAB as follows. Solutions used:

(1) 4% RG 504 PLG (Boehringer Ingelheim) in dimethyl chloride.

(2) 0.5% CTAB (Sigma Chemical Co., St. Louis, MO) in water.

In particular, the microparticles were made by combining 12.5 ml of polymer solution with 1.25 ml of distilled water and homogenizing for 3 minutes using an Omni benchtop homogenizer with a 10 mm probe at 10K rpm to form a w/o emulsion. The w/o emulsion was added to 50 ml of the 0.5% CTAB solution and homogenized for 3 minutes to form a w/o/w emulsion. The w/o/w emulsion was left stirring overnight for solvent evaporation, forming microparticles. The formed microparticles were then filtered through a 38 μ mesh, washed with water by centrifugation 4 times, and lyophilized. The microparticles were then sized in a Malvern Master sizer for future use.

Example 3: Effect of MPL and CpG Oligonucleotides on Immune Response Phenotype

Groups of 10 mice were immunized as follows: Group 1) MF59 with recombinant HIV p55 gag protein in the presence and absence of CpG oligonucleotides;

Group 2) MF59 incorporating monophosphoryl lipd A (MPL) with HIV p55 gag protein; Group 3) SDS/PLG microparticles with HIV p55 gag protein adsorbed to the surface in the presence and absence of CpG oligonucleotides; Group 4) SDS/PLG p55 adsorbed microparticles with MPLs; Group 5) recombinant protein with MPL; and Group 6) recombinant protein alone. The MF59 dose was 25 µl per animal, HIV p55 protein was 25 µg per animal, CpG oligonucleotide was 50 µg per animal, and MPL was given at 10 µg per animal. The microparticles were given at a dose containing 25 µg of protein.

MPL was obtained from Ribi Immunochem Res. Inc. (Hamilton, Montana). MPL/MF59 was prepared by dissolving MPL in $CHCl_3$, transferring the solution into Squalene/Span85 and formulating the standard MF59 emulsion with Tween80/H_2O.

Recombinant yeast p55 gag protein was produced by standard fermentation techniques well known to those skilled in the art in which yeast are disrupted by dynomill. The p55 protein was extracted from pelleted material obtained from the cell lysate in urea/NaCl buffer. The urea soluble protein was purified to >90% homogeneity by anion-exchange chromatography in the presence of 6M urea.

Mice received three intramuscular injections at weekly intervals, and serum samples were collected two weeks post third injection and assayed for total IgG (G + M + A), IgG1 and IgG2a using a chemiluminescent ELISA assay based upon CA Aequorn (Sealite Inc., Norcross, GA). Results from a typical assay are shown in Figures 1 and 2. In the case of the adsorbed microparticles, animals receiving the CpG oligonucleotides showed an IgG2a response 19-fold higher than that of the adsorbed particles alone, 7-fold higher response than adsorbed particles with MPLs, and 17-fold higher response than protein alone. In the case of the protein with MF59, animals receiving the CpG oligonucleotides showed an IgG2a response 7-fold higher than that induced in the absence of the CpG oligonucleotides, 2.6-fold higher than the combination of MF59 and MPLs, 15-fold higher than protein with MPLs, and 23-fold higher than protein alone. The results indicate that CpG oligonucleotides in combination with either MF59 or PLG microparticles stimulate a Th1 lymphocyte response which is significantly greater than the response induced by MPLs with either MF59 or PLG microparticles.

Oligonucleotides were prepared by Oligos Etc., Inc. (Wilsonville, OR). CpG1 comprises SEQ ID NO:28. CpG2 comprises the non-CpG sequence tccaggacttctctcaggtt (SEQ ID NO:29).

Example 4: IM Immunization of p55 gag Protein and Various Adjuvants

Groups of 9 mice were immunized intramuscularly, except where noted, as follows: Group 1) MF59 with recombinant HIV p55 gag protein, and DOTAP 80 in the presence of CpG1 oligonucleotide; Group 2) MF59 with recombinant HIV p55 gag protein, and DOTAP 160 in the presence of CpG1 oligonucleotide; Group 3) MF59 with recombinant HIV p55 gag protein and DOTAP; Group 4) MF59 with recombinant HIV p55 gag protein; Group 5) MF59 with recombinant HIV p55 gag protein in the presence of CpG1 oligonucleotide; Group 6) recombinant HIV p55 gag protein and DOTAP 160; Group 7) recombinant HIV p55 gag protein and CpG1 oligonucleotide; Group 8) recombinant HIV p55 gag protein, and DOTAP 160 in the presence of CpG1 oligonucleotide; and Group 9) vv-gag-pol (2×10^7 pfu) IP. The MF59 dose was 25 µl per animal, HIV p55 protein was 25 µg per animal, and CpG oligonucleotide was 50 µg per animal. Following immunization, serum anti-p55 IgG titer was measured, the results of which appear in Figure 3. As can be seen, antibody titer in the presence of a positively charged emulsion (with DOTAP) is twice as high as in the absence of a positively charged emulsion (without DOTAP). Lysis of targets (SvB cell line) by CTL was also measured with each group, the results of which appear in Figure 4. As can be seen, addition of DOTAP to result in a positively charged emulsion increases the CTL response.

What is claimed is:

1. An adjuvant composition comprising:
   a) an oligonucleotide comprising at least one CpG motif; and
   b) an oil droplet emulsion.

2. The composition of claim 1 wherein said oil droplet emulsion comprises (a) a metabolizable oil and an emulsifying agent.

3. The composition of claim 2 wherein said oil and said emulsifying agent are present in the form of an oil-in-water emulsion having oil droplets substantially all of which are less than 1 micron in diameter and wherein said composition exists in the absence of any polyoxypropylene-polyoxyethylene block copolymer.

4. The composition of claim 3, wherein said oil is an animal oil.

5. The composition of claim 3 wherein said oil is an unsaturated hydrocarbon.

6. The composition of claim 3 wherein said oil is a terpenoid.

7. The composition of claim 6 wherein said terpenoid is squalene.

8. The composition of claim 3 wherein said oil is a vegetable oil.

9. The composition of claim 3 wherein said composition comprises 0.5 to 20% by volume of said oil in an aqueous medium.

10. The composition of claim 3 wherein said emulsifying agent comprises a non-ionic detergent.

11. The composition of claim 10 wherein said emulsifying agent comprises a polyoxyethylene sorbitan mono-, di-, or triester or a sorbitan mono-, di-, or triether.

12. The composition of claim 11 wherein said composition comprises 0.01 to 0.5 % by weight of said emulsifying agent.

13. The composition of claim 3 wherein said emulsifying agent comprises a cationic detergent.

14. The composition of claim 13 wherein said cationic detergent is selected from the group consisting of hexadecyltrimethylammonium bromide, benzalkonium chloride, dimethyl dioctodecyl ammonium bromide, DOTAP, dodecyltrimethylammonium bromide, benzyldimethylhexadecyl ammonium chloride, cetylpyridinium chloride, methylbenzethonium chloride, and 4-picoline dodecyl sulfate.

15. The composition of claim 13 wherein said composition comprises 0.01 to 0.5 % by weight of said emulsifying agent.

16. The composition of claim 3 further comprising a separate immunostimulating agent.

17. The composition of claim 16 wherein said immunostimulating agent is selected from the group consisting of alum, a bacterial cell wall component, and muramyl peptide.

18. The composition of claim 1 wherein said oligonucleotide comprises at least one phosphorothioate bond.

19. The composition of claim 1 wherein said oligonucleotide comprises at least one peptide nucleic acid bond.

20. The composition of claim 18 wherein said oligonucleotide comprises a nucleotide sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NO:27, and SEQ ID NO:28.

21. The composition of claim 1 wherein said oligonucleotide comprises a CpG motif flanked by two purines immediately 5' to said motif and two pyrimidines immediately 3' to said motif.

22. The composition of claim 18 wherein said oligonucleotide comprises a nucleotide sequence selected from the group consisting of SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NO:27, and SEQ ID NO:28.

23. An immunogenic composition comprising:

a) an immunostimulating amount of an antigenic substance; and

b) an immunostimulating amount of the adjuvant of claim 1.

24. The composition of claim 23 wherein said antigenic substance is selected from the group consisting of a protein, protein-polysaccharide, protein-lipopolysaccharide, polysaccharide, and lipopolysaccharide.

25. An immunogenic composition comprising:

a) an immunostimulating amount of an antigenic substance adsorbed to poly(lactide-co-glycolide) microparticles; and

b) an oligonucleotide comprising at least one CpG motif.

26. The composition of claim 25 wherein said adsorbed antigenic substance is a recombinant protein.

27. The composition of claim 24 wherein said antigenic substance is from a virus.

28. The composition of claim 27 wherein said antigenic substance comprises a viral subunit.

29. The composition of claim 27 wherein said virus is selected from the group consisting of hepatitis C virus (HCV), hepatitis B Virus (HBV), herpes simplex virus (HSV), human immunodeficiency virus (HIV), cytomegalovirus (CMV), influenza virus (flu), and rabies virus.

30. The composition of claim 29 wherein said antigenic substance is selected from the group consisting of HSV glycoprotein gD, HIV glycoprotein gp120, and HIV p55 gag.

31. The composition of claim 24 wherein said antigenic substance is from a bacterium.

32. The composition of claim 31 wherein said bacterium is selected from the group consisting of cholera, diphtheria, tetanus, pertussis, *Helicobacter pylori*, and *Haemophilus influenza*.

33. The composition of claim 24 wherein said antigenic substance is from a parasite.

34. The composition of claim 33 wherein said parasite comprises a malaria parasite.

35. A method of stimulating an immune response in a host animal comprising administering to said animal a composition of claim 23 in an amount effective to induce an immune response.

36. The method of claim 35 wherein said host animal is a mammal.

37. The method of claim 36 wherein said mammal is a human.

38. A method of immunizing a host animal against a viral, bacterial, or parasitic infection comprising administering to said animal a composition of claim 23 in an amount effective to induce a protective response.

39. The method of claim 38 wherein said host animal is a mammal.

40. The method of claim 39 wherein said mammal is a human.

41. A method of increasing a Th1 immune response in a host animal comprising administering to said animal a composition of claim 23 in an amount effective to induce a Th1 immune response.

ABSTRACT OF THE DISCLOSURE

Adjuvant compositions which have an oligonucleotide having at least one CpG motif, and an oil droplet emulsion having a metabolizable oil and an emulsifying agent are provided. Immunogenic compositions having an immunostimulating amount of an antigenic substance, and an immunostimulating amount of an adjuvant composition are also provided. Methods of stimulating an immune response, methods of immunizing a host animal against a viral, bacterial, or parasitic infection, and methods of increasing a Th1 immune response in a host animal by administering to the animal an immunogenic composition is also provided.

Figure 1

groups	total IgG	IgG1	IgG2a
	1,156,670	1,213,515	12,337
w/CpG1	2,177,293	1,619,452	236,174
w/CpG2	1,300,018	914,905	84,834
w/MPL	1,775,948	1,441,437	32,065
P55 gag	281,840	254,977	13,562
vvgag	144,719	65,432	13,692

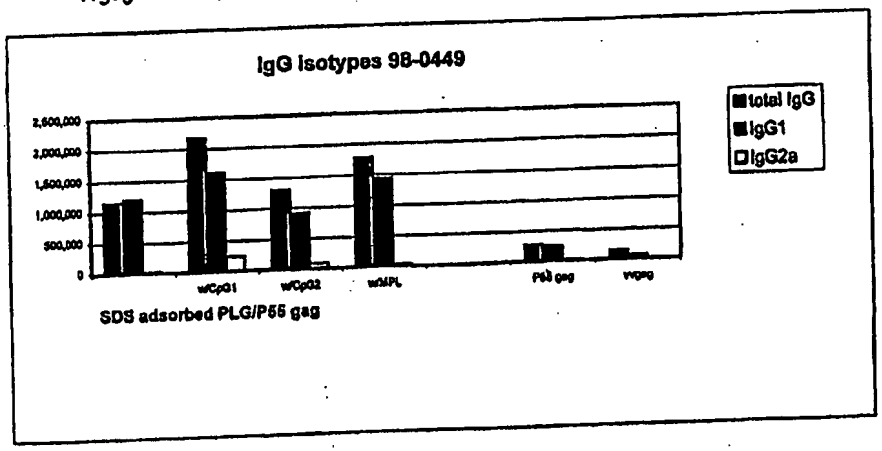
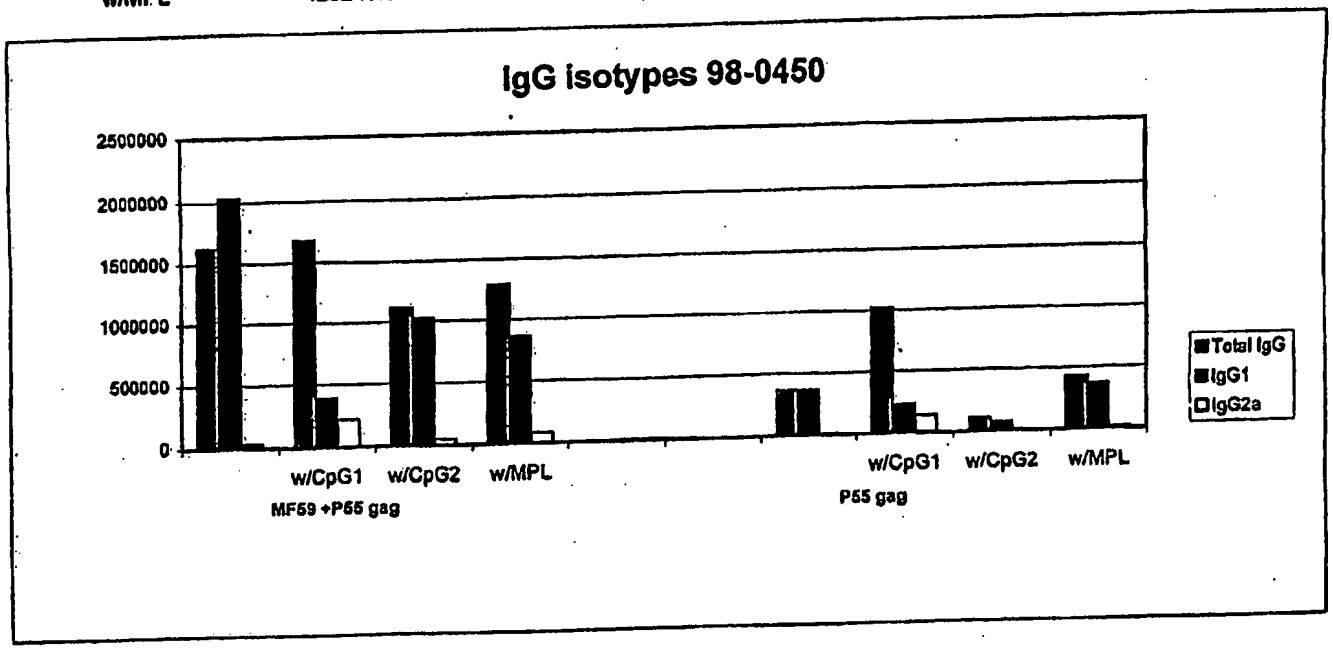


Figure 2

98-0450 groups	Total IgG	IgG1	IgG2a	IgG1/total	IgG2a/total
	1627784.5	2033181	29038	125	2
w/CpG1	1684281.5	388655	213144	23	13
w/CpG2	1119387.5	1031058	42307	92	4
w/MPL	1289791	864920	81712		
	365908.5	366661	9236	100	3
w/CpG1	1023830.5	230807	137205	23	13
w/CpG2	111442.5	75773	7029	68	6
w/MPL	426249.5	367840	14166	86	3



Serum IgG
3wp2 - 7/27/99

Group	Adjuvant	Adjuvant Dose	Antigen name & dose	Route	Plate 1	Plate 2	Average	Dilution	Titer
1	MF59/Dotap 80/CPG1	25 ul MF59 - 50 ug CPG	P55 gag protein 25 ug	IM TA	968	854	911	100	91100
2	MF59/Dotap 160/CPG1	25 ul MF59 - 50 ug CPG	P55 gag protein 25 ug	IM TA	1201	914	1058	100	105750
3	MF59/Dotap	25 ul MF59	P55 gag protein 25 ug	IM TA	408	387	398	100	39750
4	MF59-0	25 ul MF59	P55 gag protein 25 ug	IM TA	110	107	109	100	10850
5	MF59 + CPG1	25 ul MF59 - 50 ug CPG	P55 gag protein 25 ug	IM TA	569	453	511	100	51100
6	DOTAP	-	P55 gag protein 25 ug	IM TA	25	26	26	100	2550
7	CPG1	50 ug	P55 gag protein 25 ug	IM TA	25	24	25	100	2450
8	CPG + DOTAP	-	P55 gag protein 25 ug	IM TA	52	50	51	100	5100
9	No Adjuvant	-	vvgag pol 1x10^7	IP	7	7	7	100	700

Figure 3

p55 protein with MF59/DOTAP/CPG Formulations

Group Antigen Adjuvant Route	Relevant		
	TARGET RATIO	SvB p7g	SvB gag^B
1 MF59/Dotap 80/CPG1 p55 protein (25 ug) IM TA	60 15 4	30 11 2	-2 -2 -2
%Spon. Release		12	11
2 MF59/Dotap 160/CPG1 p55 protein (25 ug) IM TA	60 15 4	49 21 [illegible]	4 1 0
3 MF59/Dotap p55 protein (25 ug) IM TA	60 15 4	23 7 5	2 0 0
4 MF59-0 p55 protein (25 ug) IM TA	60 15 4	13 2 -1	1 0 -2
5 MF59 + CPG1 p55 protein (25 ug) IM TA	60 15 4	15 4 0	0 0 0
6 DOTAP p55 protein (25 ug) IM TA	60 15 4	46 15 3	4 0 -1
7 CPG1 p55 protein IM TA	60 15 4	23 7 0	-2 -2 -2
8 CPG+ DOTAP p55 protein (25 ug) IM TA	60 15 4	28 11 4	1 0 -1
9 vv-gag-pol $2x10^7$ pfu IP	60 15 4	70 31 19	1 -1 -1

Figure 4